Identification of Molecular and Cellular Responses of *Desulfovibrio vulgaris* Biofilms under Culture Conditions Relevant to Field Conditions for Bioreduction of Heavy Metals

MIAMI UNIVERSITY
OXFORD OHIO

Possible Roles of Extracellular Protein and the Megaplasmid in the Formation of *Desulfovibrio vulgaris* Biofilms

M. E. Clark[1], J.D. Wall[2], Z. He[3], J. Zhou[3], J. Keasling[4], and M. W. Fields[1]

[1]Department of Microbiology, Miami University, Oxford, OH; [2]Department of Biochemistry, University of Missouri, Columbia, MO; [3]Institute for Environmental Genomics, Universtiy of Oklahoma, Norman, OK; [4]Synthetic Biology, Lawrence Berkeley National Laboratory, Berkeley, CA

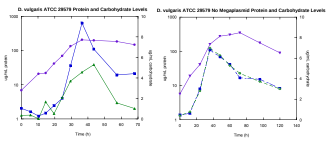
Abstract:

Desulfovibrio vulgaris ATCC29579 is a sulfate reducing bacterium that is commonly used as a model for direct and indirect heavy metal reduction, and can also be a causitative agent of metal corrosion. **Objective:** Characterize *D. vulgaris* biofilms and identify key proteins necessary for biofilm formation and maintenance. **Results**: During growth with lactate and sulfate, internal carbohydrate levels increased throughout exponential phase, and peaked as the cells transitioned to stationary phase. The carbohydrate to protein ratio (C:P) peaked at 0.05 ug/ug as the cells transitioned to stationary phase, and then declined to 0.02 ug/ug during extended stationary phase. In contrast, a strain of *D. vulgaris* that does not contain the megaplasmid (rmp), maintained higher internal carbohydrate levels and the C:P ratio peaked 2fold higher compared to wild-type. The C:P ratio in extended stationary phase was 4fold higher compared to the wild-type. Under the tested growth conditions, we observed biofilm formation in wild-type cells, but the rmp strain formed less biofilm (2fold decrease). In addition, carbohydrate levels in the culture supernatant were approximately 2fold increased for wild-type cells compared to rmp cells. We hypothesized that carbohydrate was reallocated to the external cell proper for biofilm formation. However, biofilm contained little carbohydrate (0.6 to 1.0 ug/ml) and had a similar C:P ratio compared to wild-type early stationary-phase cells. Staining with calcafluor white also indicated the presence of little external carbohydrate in *D. vulgaris* biofilms. The formation of biofilm was hindered by the presence of protinease K, trypsin, and chymotrypsin, however, the growth of planktonic cells was not. In addition, when *D. vulgaris* biofilm was treated with a protease, biofilm was degraded. In comparison, the biofilm of *Shewanella oneidensis* contained more carbohydrate, and the *S. oneidensis* biofilm was not significantly affected by protease treatment. Electron micrographs indicated the presence of filaments between the biofilm cells, and filaments were susceptible to protease degradation. Biofilm filtrates contained soluble protein, and SDS-PAGE analysis suggested different polypeptide profiles between filtrates, planktonic, and biofilm samples. The results indicated that *D. vulgaris* changes carbohydrate distributions in response to growth phase, the megaplasmid contains genes important for carbohydrate distribution and biofilm formation, and *D. vulgaris* biofilms contain extracellular filaments that may be important for the initial stages of biofilm formation.

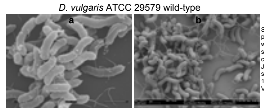
Introduction:

The work presented here involves *D. vulgaris* ATCC 29579 and the Δmegaplasmid strain of this organism which is lacking the 0.2 Mb plasmid. Some of our initial work has shown that *D. vulgaris* increase carbohydrate production as it transitions from log to stationary phase. Previous studies have demonstrated that microorganisms can increase glycogen just before stationary phase. Our previous work indicated that *D. vulgaris* does not maintain all of this carbohydrate internally and excreted polysaccharide might be used for biofilm production. Work with different *Desulfovibrio* spp has shown that biofilms will be produced by these organisms and that reduction properties differ from their planktonic counterparts (Dunsmore et al., 2002; Beyenal et al., 2004; Beyenal and Lewandowski, 2004). Little is known about the cellular composition of *Desulfovibrio* spp biofilms, although it is known that cell clusters can be observed and that the biofilm can increase thickness and become porous (Dunsmore, et al., 2002; Beyenal and Lewandowski, 2004). Beech et al (1991) demonstrated that *Desulfovibrio desulfuricans* can produce EPS, with measurable amounts of neutral hexose, uronic acid, and carbohydrates like glucose, mannose, and galactose when grown on steel surfaces. In this study, we demonstrate that *D. vulgaris* does not produce a significant amount of EPS to go towards biofilm formation, and that initial biofilm formation may be dependent upon proteinaceous material.

Results:

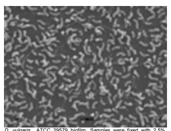


D. vulgaris ATCC 29579 wild-type



Scanning electron images of *D. vulgaris* ATCC 29579 planktonic (**a**) and biofilm (**b**) cells. Samples were fixed with 2.5% gluteraldehyde, 2.0% paraformaldehyde, 0.05M sodium cacodylate followed by ethanol dehydration and critical point drying. Planktonic images were taken on a JOEL 840a SEM at a working distance of 8mm, aperature setting of 4, and a probe current of $6x10^{-11}$, and a keV of 19. Biofilm images were taken on a Zeiss Supra 35 FEG-VP SEM.

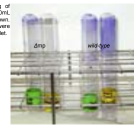
During transition from log into stationary phase, *D. vulgaris* wild-type cultures peak in carbohydrate production to about 10ug/mL. Approximately half of this carbohydrate is found internally during this transition. Measurements of culture supernatant revealed that although not all the carbohydrate was accounted for, there was an increased level of carbohydrates within the supernatant, opening up the possibility that this carbohydrate may be going towards biofilm production. Biofilm formation begins approximately 20-25h into growth, just as carbohydrate production begins to increase. In contrast, the Δmegaplasmid strain produced less carbohydrate overall (~7ug/mL) that mainly remained internal as the cell transitioned from log to stationary phase. Electron images of wild-type cells showed that planktonic cells have a smoother appearance than the biofilm cells. In addition, fewer filaments were observed in biofilm cells compared to planktonic cells. The 'filaments' appeared to have more structural integrity within the biofilm and may play a role in initial formation and/or structure of the biofilm matrix. Although the decrease in carbohydrate production coincided with the formation of biofilm, evidence for an exopolysaccharide material was not measured or observed.



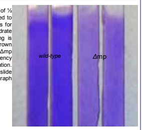
D. vulgaris ATCC 29579 biofilm. Samples were fixed with 2.5% gluteraldehyde, 2.0% paraformaldehyde, and 0.05M sodium cacodylate proceeded by ethanol dehydration and critical point dried. Images were taken on a JEOL 840a SEM with a working distance of 8mm, apeture setting of 4, probe current of $6X10^{-11}$, and a keV of 19.

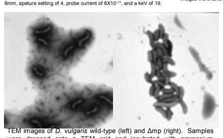
D. vulgaris Δmegaplasmid biofilm. Samples were fixed with 2.5% gluteraldehyde, 2.0% paraformaldehyde, and 0.05M sodium cacodylate proceeded by ethanol dehydration and critical point dried. Images were taken on a Zeiss Supra 35 FEG-VP SEM.

Crystal violet staining of culture tubes after 10mL cultures were grown. Adhered cells were stained with crystal violet.

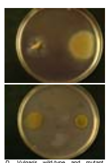


Crystal violet staining of ½ microscope slides used to collect biofilm samples for protein and carbohydrate analysis. Less staining is seen on the slides grown in the presence the Δmp strain indicate a defiency in biofilm formation. Quantitation of these slide is dipicated in the graph below.

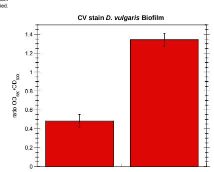


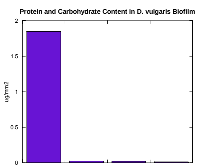


TEM images of *D. vulgaris* wild-type (left) and Δmp (right). Samples were dropped onto a TEM grid and incubated with ammonium molybdate. Specidmans were then viewed with a JEOL 100S TEM



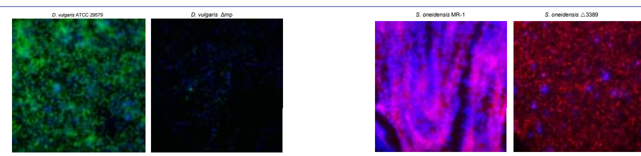
D. Vulgaris wild-type and mutant grown on 0.3%(top) and 1%(bottom) LS4D agar plates.



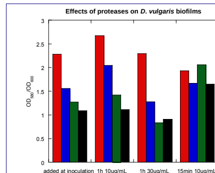


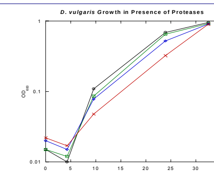
Crystal violet staining of *D. vulgaris* biofilms showed a confluent structure adhered to glass tubes or slides for the wild-type cells. The Δmegaplasmid strain produced less biofilm as observed by visual inspection and quantification with crystal violet stain. Quantification of the crystal violet stain showed almost 3-fold less biofilm produced by the Δmegaplasmid strain when compared to wild-type cells. *D. vulgaris* biofilm contained an significant amount of protein, but carbohydrate levels were low even when different assays specific for pentoses and hexoses were used. These results indicated that the significant portions of the carbohydrate spike that is depleted as *D. vulgaris* enters stationary phase does not go to the formation of biofilm.

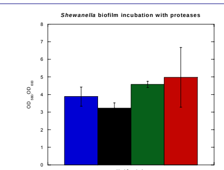
Electron micrographs of the wild-type biofilm verses the Δmegaplasmid strain showed significantly less cells attached to the glass slides for the Δmegaplasmid strain. The Δmegaplasmid strain appears to have fewer flagella, as visualized with TEM. Also, the mutant strain is deficient in motility compared to the wild-type, which may be due to a lack of flagella. This lack of flagella in the Δmegaplasmid strain may be the reason why we see fewer cells attaching when viewing biofilms with SEM. The Δmegaplasmid cells also did not appear to have the 'filaments' that were observed in the wild-type biofilms. Although previous research with other organisms have suggested that 'filaments' similar to the ones observed here might be dehydrated carbohydrate that is part of the matrix, our results indicated that little carbohydrate can be measured in biofilm samples and that little can be visualized with fluorescent staining.



D. vulgaris biofilms were stained with calcofluor white (blue) to analyze for polysaccharides and DAPI (green) to observe cells. Images suggested that even though there is an abundance of cells for wild-type biofilms, little polysaccharide is present in the matrix. The lack of carbohydrate production in the Δmegaplasmid strain is not surprising due to the lack of few adhered cells. Whether the Δmegaplasmid strain is unable to reach the surface or whether it is unable to adhere is still unknown. The lack of polysaccharide within *D. vulgaris* wild-type biofilms is apparent when compared with a control, such as *Shewanella oneidensis*. The MR-1 strain of *S. oneidensis* does contain an abundance of polysaccharides within the matrix of its biofilm, which is visible with the calcoflour white stain (shown in blue). MR-1 was also stained with Acridine Orange to determine the number of cells (shown in red). The mutant, *S. oneidensis* △3389 is defective in biofilm formation, and calcofluor white does not show significant staining. This is seen by the lack of polysaccharide present within the biofilm. These results further confirm that *D. vulgaris* does not produce a typical biofilm that is composed of exopolysaccharides.

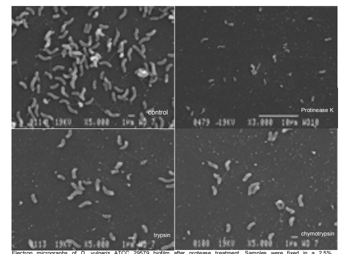




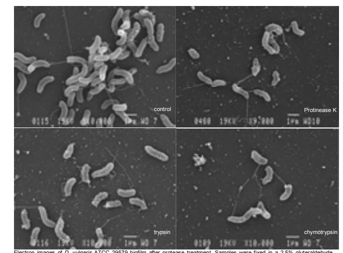


After incubation with a protease, *D. vulgaris* biofilms appear to be less confluent as indicated by crystal violet staining (OD_{580}/OD_{600} ratio). When added at the time of culture inoculation, the protease inhibited biofilm formation but did not hinder planktonic growth. When samples were taken from a late log phase culture and incubated for 15 min or 1 h in the presence of a protease, biofilm was degraded as indicated by the decrease in OD_{580}/OD_{600} ratio. Degradation did not appear to be dose dependent. As a control, when a *Shewanella oneidensis* biofilm was treated in the same fashion, little degradation was observed and the biofilm remained intact.

Red, control; blue, protinease K treated; green, trypsin treated; black, chymotrypsin treated.



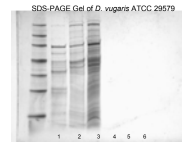
Electron micrographs of *D. vulgaris* ATCC 29579 biofilm after protease treatment. Samples were fixed in a 2.5% gluteraldehyde, 2.0% paraformaldehyde, 0.05M sodium cacodylate solution, followed by ethanol dehydration and critical point drying. Images were taken on a JOEL 840a SEM with a working distance of 8mm, probe current of $6x10^{-11}$, apeture of 4, and keV of 19. Images clockwise from top left hand corner: Control, protinease K, chymotrypsin, trypsin treament. Slides were treated in a solution of 50mM PBS pH 7.2 with10ug/mL of enzyme for 1h.



Electron images of *D. vulgaris* ATCC 29579 biofilm after protease treatment. Samples were fixed in a 2.5% gluteraldehyde, 2.0% paraformaldehyde, 0.05M sodium cacodylate solution, followed by ethanol dehydration and critical point drying. Images were taken on a JOEL 840a SEM with a working distance of 8mm, probe current of $6x10^{-11}$, apeture of 4, and keV of 19. Images clockwise from top left hand corner: Control, protinease K, chymotrypsin, trypsin treament. Slides were treated in a solution of 50mM PBS pH 7.2 with10ug/mL of enzyme for 1h.

D. vulgaris ATCC 29579 biofilm was shown to be susceptible to protease degradation with three different proteases: protinease K, trypsin, and chymotrypsin. Control samples incubated for one hour in 50 mM PBS pH7.2 remain adhered to the glass slide with no apparent cell loss or degradation of the 'filament' structures. Far fewer cells remained after protease treatment, and chymotrypsin appeared to have the most effect. Some of the filament structures do remain after treatment, but appear more degraded than those in the control samples. Damage to the external cell proper was also apparent in some images. Biofilms incubated for only 15 min and those incubated at a higher concentration of protease (30ug/mL) had a similar appearance to the images shown here. When the protease was added at the time of inoculation, biofilm formation was hindered, with protinease K appearing to have more of an affect that resulted in less adhered cells compared to both trypsin-treated and the control. These results indicated that proteases hindered biofilm formation as well as affected biofilm stability. The degradation of the 'filaments' indicated that these structures are proteinaceous in nature and may play a role in the formation and stability of *D. vulgaris* biofilms. Flagella are known to assist in biofilm formation by giving the cells a means of reaching the surface for attachment. It may be possible that *D. vulgaris* not only uses flagella as a means to reach the glass slide but also as part of biofilm matrix. Further analysis of these structures will be done in order to determine if they may be flagella and what possible role they may play.

SDS-PAGE Gel of *D. vugaris* ATCC 29579



Lane 1: MW markers, Lane 2: Biofilm filtrate (4.2 mg)*, Lane 3: Biofilm supernatant (10.0 mg), Lane 4: Planktonic supernatant (10.1 mg), Lane 5: anaerobic planktonic filtrate (2.0 mg)**, Lane 6: aerobic planktonic filtrate**.

*amount of protein loaded for each sample

**very little/no protein concentration measured with Bradford assay

DVU #	Size	Possible Protein	Function
DVU197 6	548a a	chaperonin 60kDa (547aa)	GroEL-like chaperone
DVU323 6	548a a	GTP binding protein HflX	
DVU114 7	459a a	alginate o-acetyltransferase	Predicted membrane protein involved in D-alanine export IPR004299: Membrane bound O-acyl transferase IPR008980: Viral capsid/hemagglutinin protein
DVU250 2	296a a	UDP-N-acetylenolpyruvoyl -glucosamine reductase	Members of this family are UDP-N-acetylenolpyruvoylglucosamine reductase enzymes 1.1.1.158 , which are also called UDP-N-acetylmuramate dehydrogenases. They are a part of the pathway for the biosynthesis of the UDP-N-acetylmuramoyl-pentapeptide, which is a precursor of bacterial peptidoglycan.

Table 1: Examples of proteins that correspond to the approximate size of the peptide that appear in the filtrate sample.

SDS-PAGE 1-D gel electrophoresis indicated that there was not a striking difference between the planktonic and biofilm polypeptide profiles. Biofilm samples were also collected and filtered to analyze for proteins that may be present within the biofilm matrix (extracellular). The filtrate (Lane 2) showed a reduced amount of bands, but the majority of bands were observed in the biofilm cell profile. However, 5 to 6 polypeptide bands appeared to be enriched in the filtrate fraction. The estimated sizes of these polypeptides were determined and compared to estimated proteins from the genome sequence (Table 1). Possible polypeptides in the biofilm filtrate included presumptive chaperones, binding proteins, export proteins, and peptidoglycan synthesis. More detailed analyses are underway.

Materials & Methods:

Growth: D. *vulgaris* and Δmegaplasmid strains were grown in batch tubes containing LS4D minimal medium at 30°C. Biofilm samples were grown on glass slides that were inserted into the tube.

Biofilm Quantification: Cultures were removed from the tubes and the biofilm samples were rinsed once with 50 mM PBS pH 7.2. Biofilms were stained for 5min with crystal violet and then rinsed with water. Biofilm samples were then soaked in 10mL of an 80% ethanol/20% acetone solution to remove the crystal violet. The extract solution was read at an OD of 580 and the OD_{580}/OD_{600} ratio was calculated.

Protein & Carbohydrate Calorimetric Assays: Protein was determined by method of Lowry or Bradford. Hexoses were determined by the cysteine-sulfuric acid method. Pentoses and uronic acid was measured by the ferric-orcinal assay and carbazole assay, respectively.

Fluorescent Staining for EPS: A 10 mg/mL solution of calcofluor white was domed over *D. vulgaris* biofilm that had formed on glass slides. The slide was kept in the dark and incubated at room temperature for 15 min. For the last 5 min, acridine orange was added. The slide was rinsed 3X with PBS and viewed on an Olympus AX-70 Multimode Microscopy System with a DAPI filter and an acridine orange filter to view the calcofluor white and acridine orange, respectively.

Protease Treatments: Biofilm samples were grown on glass slides and harvested after approximately 30 h of growth. Samples were then rinsed with 10 mL of 60 mM PIPES and a 50 mM PBS pH 7.2 solution was added to enough volume to cover the glass slide. The PBS contained 10 ug/mL or 30 ug/mL of either protinease K, trypsin, chymotrypsin, or no protease (control). Biofilm samples were incubated for 15 min or 1h with the protease. After incubation, slides went directly into crystal violet for biofilm quantification or gluteraldehyde/paraformaldehyde fixative for SEM preparation. *D. vulgaris* cultures were also treated with the three proteases (separately) during growth via the addition of the protease at time of inoculation.

SDS-PAGE Electrophoresis: Samples were prepared as follows: each sample was diluted to 37.5 μL to desired protein concentration and added to 10 μL of 4X loading buffer and 2.5 μL of 1 M DTT. Samples were heated at 100°C for 5 min and centrifuged for 3 min at 6,000 rpm. Samples were loaded on a 4-20% gradient gel (Life Therapeutics) and electrophoresed (approximately 50 V). Gels were stained with Coomassie Blue.

Conclusions:

- *D. vulgaris* ATCC 29759 increased carbohydrate production into stationary phase
- *D. vulgaris* wild-type produced more carbohydrate than the Δmegaplasmid strain but the carbohydrate did not appear to be internal as compared to the Δmegaplasmid
- The Δmegaplasmid strain was deficient in biofilm formation compared to wild-type
- Wild-type biofilms contained measurable amounts of protein but did not contain large amounts of carbohydrate
- *D. vulgaris* biofilms contained little hexoses, pentoses, or uronic acid.
- The lack of significant amounts of carbohydrate was confirmed with calcofluor white stain
- Wild-type biofilms contained 'filaments' that were uniform in diameter and appeared to be proteinaceous in nature
- *D. vulgaris* biofilms can be inhibited as well as degraded in the presence of a proteases
- 'Filaments' within the biofilm were degraded by proteases and may play a role in biofilm formation and stability
- Biofilm filtrate samples appeared to be enriched for particular polypeptides and further analysis of the filtrates may identify proteins important for biofilm formation and/or stability

This work was funded by the Environmental Remediation Sciences program under the U.S. Department of Energy, Office of Science.